ATTORNEY DOCKET NO. 14014.0349U2
PATENTIN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)

Blackshear *et al.*)

Group Art Unit: 1634)

Application No. 10/049,586)

Examiner: Sisson, B. L.)

Filing Date: February 12, 2002)

Confirmation No. 9700)

For: TTP-RELATED ZINC FINGER)

DOMAINS AND METHODS OF USE)

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

NEEDLE & ROSENBERG, P.C.
Customer Number 36339

Sir:

I, Jack D. Keene, hereby declare that:

1. I am a James B. Duke Professor of Molecular Genetics and Microbiology and Director of the Center for RNA Biology at Duke University. I hold a Ph.D. in microbiology and immunology from University of Washington, Seattle and a B.A. from University of California, Riverside. I was trained in the molecular biology of RNA viruses as a Staff Fellow at the National Institutes of Health in Bethesda, Maryland. I have over 30 years experience in the field of RNA metabolism, with an emphasis on RNA-protein interactions, and over 25 years experience in the study of regulation of RNA by RNA-binding proteins. This includes specific experience in the study of the structure and function of RNA-binding proteins and their role in gene expression. A partial curriculum vitae is attached to this declaration as an exhibit.

2. I have reviewed the specification and the claims in the above-identified application. Specifically, I have reviewed page 30, beginning at line 8, wherein the Applicants disclose that

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"[a] variety of assay methods can be used to determine whether a given compound interferes with TTP or related protein binding to the GM-CSF ARE and the breakdown of GM-CSF mRNA."

3. As an expert in the field of RNA metabolism in general and the study of regulation of RNA by RNA-binding proteins in particular, and as an individual with extensive knowledge of the level of understanding of those of skill in the art of RNA metabolism at the time Application Serial No.10/049,586 was filed, I believe that someone in the field of RNA metabolism at the time the application was filed would have been able to envision all of the steps of the recited methods for identifying a compound that interfered with the binding of TTP to an ARE based on the description provided in the specification.

4. Further, it is my opinion that the description of the assay methods that can be used in the provided methods is clear and straightforward. It is my opinion that a person in this field would have recognized phrases such as "would include," "could use," and "would probably be the most convenient" as a sufficiently affirmative and clear disclosure of the types of assay methods available to a practitioner at the time the application was filed to allow for the practice of the claimed method. Further, the example of the assay methods provided on pages 64-68 of the specification, in light of the specification as a whole, gives a clear indication of what the claimed methods are and how they are to be practiced.

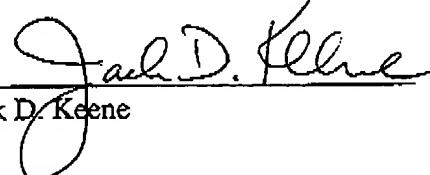
5. I further believe that the asserted use of the claimed method, *i.e.*, for the identification of a compound that inhibits the degradation of GM-CSF mRNA that would be a candidate for use in a method of treating granulocytopenia in a subject, is a scientifically credible utility based on the data presented in the specification. For example, data are provided on pages 61-106 of the specification, such as for example page 100, lines 9-19, demonstrating the degradation of the

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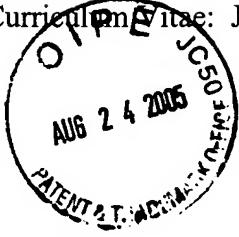
TNF- α mRNA ARE by TTP. Further, the data provided on page 38, lines 14-22, demonstrating the accumulation, prolonged expression, and lack of the deadenylated form of GM-CSF mRNA in TTP-deficient cells in response to stimulus, indicate the degradation of GM-CSF mRNA by TTP. These data, in view of the known role of GM-CSF in granulocytopenia (Nemunaitis J, Drugs. 1997 Nov;54(5):709-29, attached), provide a clear and credible indication that an inhibitor of GM-CSF mRNA degradation could be identified for use in treating granulocytopenia.

6. All statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 8/22/05



Jack D. Keene



CURRICULUM VITAE

NAME: Jack D. Keene

CURRENT POSITION: James B. Duke Professor of Microbiology
Box 3020
Duke University Medical Center
Durham, North Carolina 27710

DATE OF BIRTH: June 21, 1947

TELEPHONE NUMBER: (919) 684-5138/fax (919) 684-8735

SOCIAL SECURITY NUMBER: available on request

MARITAL STATUS: Married: Judy May Keene, Landscape Painter
Children: Michael Ryan K. and Lisa Erin K.

EDUCATION: University of California, Riverside, A.B. 1969, Biology

University of Washington, Seattle, Ph.D. 1974
Microbiology and Immunology (Research Advisor:
Helen R. Whiteley) Dissertation: *Enzymatic Control of
Histone Phosphorylation and Dephosphorylation During
the Cell Cycle of Physarum polycephalum.*

PROFESSIONAL AND UNIVERSITY AFFILIATIONS:

2002-present James B. Duke Professor
 Department of Molecular Genetics and Microbiology
 Director, Center for RNA Biology
 Duke University Medical Center

1992-2002 James B. Duke Professor and Chairman
 Department of Microbiology, D.U.M.C.

1995-2003 Director of Basic Science Research
 Duke Comprehensive Cancer Center

1988 - 1992 Professor, Department of Microbiology and Immunology,
 D.U.M.C.

Curriculum Vitae: Jack D. Keene

1984 - 1988	Associate Professor, Dept of Microbiology and Immunology D.U.M.C.
1985-present	Associate Research Professor (secondary appointment) Division of Rheumatology and Clinical Immunology Department of Medicine, D.U.M.C.
1979 - 1984	Assistant Professor, Department of Microbiology and Immunology, D.U.M.C.
1974 - 1978	Staff Fellow, Laboratory of Molecular Genetics, NINDS, National Institutes of Health, Bethesda, Maryland (Robert A. Lazzarini)

AWARDS AND HONORS:

American Cancer Society, Faculty Research Award (1981-86)
Nanoline Duke Faculty Scholar (1981-84)
Arthritis Foundation, Devil's Bag Award (1985-88)
Pew Scholar in the Biomedical Sciences (1986-1990)
Fellow of the American Academy of Microbiology
James B. Duke Distinguished Professorship
Member of the Henry Kunkel Society

SCIENTIFIC SOCIETIES AND PROGRAMS:

American Society for Microbiology (ASM:1970-present)
American Society for Virology (ASV:1981-present)
American Society for Biochemistry and Molecular Biology (FASEB:1984-present)
The RNA Society [1994-present]
Member: Duke University Comprehensive Cancer Center; Program in Genetics; Program in Cellular and Molecular Biology; Program in Molecular Cancer Biology

EDITORIAL SERVICE:

Associate Editor of Virology (1983-present)
Editorial Board: Molecular and Cellular Biology (1991-present)
Editorial Board: Journal of Biological Chemistry (2003-present)
Editorial Board: Journal of Virology (1985-1995)
Editorial Board: Molecular Diversity (1995-2002)
Editor of Microbiology and Molecular Biology Reviews (1992-2000)
Alliance for Cell Signaling (2001-present)
Editor in Chief: Microbiology and Molecular Biology Reviews (2000-2005). *The number one impact journal in the field of microbiology for past four years.*

Curriculum Vitae: Jack D. Keene

NATIONAL SERVICE: (numerous *ad hoc* review panels)

- External Review Committee: Department of Biochemistry, Microbiology and Molecular Genetics, University of Cincinnati (2002)
- Policy Conference Plenary Speaker: National Institutes of Health Office of Biotechnology Activities: "Institutional Biosafety Committees in a Changing Research Landscape" (2001-03)
- National Cancer Institute Review Panel for Molecular Targeting Laboratories (2001)
- External Review Committee: Department of Microbiology and Immunology, Vanderbilt University School of Medicine (2000)
- External Review Committee: Department of Microbiology and Immunology, University of Virginia School of Medicine (2000)
- Intramural Review Committee: NCI, Laboratory of Molecular Biology (1999)
- Intramural Review Committee: NICHD, Laboratory of Eukaryotic Transcription (1996)
- NCI site visit team: Memorial Sloan-Kettering Cancer Center grant (1997 and 2002)
- Intramural Review Committee: NICHD, Laboratory of Molecular Genetics (1995)
- National Selection and Advisory Board-***PEW Scholars in the Biomedical Sciences*** (1991-96)
- Chairman, Molecular Biology Study Section, NIH (1993-95)
- Member, Molecular Biology Study Section, NIH (1990-95)
- Arthritis Foundation Fellowship Committee (1990-1992)
- Arthritis Foundation Research Committee (Scientific Council, 1990-1992)
- Member, Experimental Virology Study Section, NIH (1984-1988)

INSTITUTIONAL SERVICE

- Role in dozens of committees of institutional evaluation, personnel assessment, programmatic initiatives and faculty searches, in addition to ten years of service as departmental chair.
- Twenty-five years as chair of the Duke University Biosafety Committee.
- Member of the Provost's Advisory Committee on Distinguished Professors and chair of the Medical Center Subcommittee.

MAJOR SPONSORED RESEARCH: 1979-present

- NIH/NIAID: R01 AI16099 (1979-93) *Replicative Mechanisms of the Neg. Strand RNA Viruses.*
- NIH/NCI: P01 CA30246 (1981-93) *Regulatory Functions of Protein-Nucleic Acid Interactions.*
- NIH/NCI: R01 CA60083 (1993-1998) *Determinants of Specificity in Protein-RNA Interactions.*
- NIH/NCI: R01 CA79907 (1999-2006) *Functions of Mammalian ELAV RNA-Binding Proteins.*
- NIH/NIAID: R01 AI16099 (2000-2006) *Post-transcriptional Regulation of T-Cell Activation.*
- NIH/NCI: Co-Investigator with Daniel Kenan, R21/R33: CA94365 (2002-2007)
Endothelial Cell Molecular Alterations in Cancer

BIOTECHNOLOGY EXPERIENCE

- Autoimmune diagnostic licenses and consultation, *MBL, Inc.*, Nagoya, Japan (1991-pres)
- Consultant, *BioWhittaker, Inc.*, Walkersville, MD., *LipoGen, Inc.*, Knoxville, TN. (1985-91)
- Scientific Advisory Board, *Message Pharmaceuticals, Inc.*, Malvern, P.A. (1998)
- Co-founder, *SARCO, Inc.*, a combinatorial chemistry company acquired by *Pharmaceutical Product Development, Inc.* in 1996 (www.ppd.com)
- Founder, *Combinatorial Sciences Systems*, Durham, N.C. (IP holding company)
- Founder, *Ribonomics, Inc.*, Research Triangle Park, N.C. (www.ribonomics.com or www.transcriptome.com)

PATENT PORTFOLIOS:

"Methods and Compositions Useful in the Diagnosis and Treatment of Autoimmune Disease"
U.S. Patent 4,751,181 issued June 1988; U.S. Patent 5,541,291 issued July 1996; U.S. Patent 5,721,110 issued February 1998; European Patent EPO-0205579 issued February 1996; Japanese Patent 2680811 issued September 1997; European Patent EPO-0690307 issued January 1998.

"Protein Sequence Domains for the Specific Recognition and Binding to RNA and for Control of its Processing and Expression" U.S. Patent 5,561,222 issued October 1996; U.S. Patent 5,866,680 issued February 2, 1999.

"Methods and Compositions Useful in the Recognition, Binding and Expression of Ribonucleic Acids Involved in Cell Growth, Neoplasia and Immunoregulation" U.S. Patent 5,444,149 issued August 1995; U.S. Patent 5,525,495 issued June 1996; U.S. Patent 5,698,427 issued December 16, 1997, U.S. Patent 5,773,246 issued June 30, 1998.

"Method of Isolating Ribotypes and Proteotypes" U.S. Patent 5,882,866, issued March 1999; U.S. Patent 5,972,620 issued October 26, 1999.

"Methods for Making Epitopes" U.S. Patent 6,582,902, issued June 24, 2003.

"Methods for Isolating and Characterizing Endogenous mRNA-Protein (mRNP) Complexes"
U.S. Patent 6,635,422 issued October 21, 2003, and world-wide patents pending.

SELECTED PUBLICATIONS: chronologically organized

Keene, J.D. and Lazzarini, R.A. (1976) A comparison of the extents of methylation of vesicular stomatitis messenger RNA. Virology 69: 364-367.

Keene, J.D., Rosenberg, M. and Lazzarini, R.A. (1977) The characterization of the 3' terminus of RNA isolated from vesicular stomatitis virus and its defective interfering particles. Proceedings of the National Academy of Sciences (USA) 74: 1353-1357.

Colonna, R.J., Lazzarini, R.A., **Keene, J.D.** and Banerjee, A.K. (1977) In vitro synthesis of messenger RNA by a defective interfering particle of vesicular stomatitis virus. Proceedings of the National Academy of Sciences (USA) 74: 1884-1888.

Keene, J.D., Schubert, M., Rosenberg, M. and Lazzarini, R.A. (1978) *A comparative study of nucleotide sequences at the 3' termini of RNA from vesicular stomatitis virus and its defective interfering particles.* In: Persistent Viruses, G. Todaro, J. Stevens, C.F. Fox, eds. Academic Press, New York.

Keene, J.D., Schubert, M., Lazzarini, R.A. and Rosenberg, M. (1978) Nucleotide sequence homology at the 3' termini of RNA from vesicular stomatitis virus and its defective interfering particles. Proceedings of the National Academy of Sciences (USA) 75: 3225-3229.

Schubert, M., **Keene, J.D.**, Lazzarini, R.A. and Emerson, S.U. (1978) The complete sequence of the in vitro transcription product from a defective interfering particle of vesicular stomatitis virus. Cell 15: 102-112.

Keene, J.D., Schubert, M. and Lazzarini, R.A. (1979) Terminal sequences of vesicular stomatitis virus RNA are both complementary and conserved. Journal of Virology 32: 167-174.

Schubert, M., **Keene, J.D.** and Lazzarini, R.A. (1979) A specific internal RNA polymerase recognition site of VSV RNA is involved in the generation of DI particles. Cell 18: 749-757.

Li, J.K.-K., **Keene, J.D.**, Scheible, P.P. and Joklik, W.K. (1980) *Terminal sequence homologies in reovirus genes.* In: Virus Genetics, B.N. Fields, R. Jaenisch, C.F. Fox, eds., Academic Press, New York.

Schubert, M., **Keene, J.D.**, Herman, R.C. and Lazzarini, R.A. (1980) The site on the VSV genome specifying polyadenylation and the end of the L gene mRNA. Journal of Virology 34: 550-559.

Keene, J.D., Schubert, M. and Lazzarini, R.A. (1980) Intervening sequence between the leader region and the nucleocapsid gene of VSV RNA. Journal of Virology 33: 789-794.

Curriculum Vitae: Jack D. Keene

Li, J.K.-K., **Keene, J.D.**, Scheible, P.P. and Joklik, W.K. (1980) Nature of the 3'-terminal sequences of the plus and minus strands of the SI gene of reovirus serotypes 1, 2 and 3. Virology 105: 41-51.

Li, J.K.-K., Scheible, P.P., **Keene, J.D.** and Joklik, W.K. (1980) The plus strand of reovirus gene S2 is identical with its in vitro transcript. Virology 105: 282-286.

Herman, R.C., Schubert, M., **Keene, J.D.** and Lazzarini, R.A. (1980) Polycistronic vesicular stomatitis virus transcripts. Proceedings of the National Academy of Sciences (USA) 77: 4662-4665.

Keene, J.D., Piwnica-Worms, H. and Isaac, C.L. (1981) *Structure and origin of terminal complementarity in the RNA of DI-LT(HR) and sequence arrangements at the 5' end of VSV RNA.* In: The Replication of Negative Strand Viruses, D.H.L. Bishop and R.W. Compans, eds., Elsevier North Holland, Inc., New York.

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Isaac, C.L. and **Keene, J.D.** (1981) Transfer RNAs associated with vesicular stomatitis virus. Journal of General Virology 56: 141-151.

Keene, J.D., Thornton, B.J. and Emerson, S.U. (1981) Sequence specific contacts between the RNA polymerase of vesicular stomatitis virus and the leader RNA gene. Proceedings of the National Academy of Sciences (USA) 78: 6191-6195.

Lazzarini, R.A., **Keene, J.D.** and Schubert, M. (1981) A review: the origins of defective interfering particles of the negative strand RNA viruses. Cell 26: 145-154.

Lazzarini, R.A., Chien, I., Yang, F. and **Keene, J.D.** (1982) The metabolic fate of independently initiated VSV mRNA transcripts. Journal of General Virology 58: 429-441.

Isaac, C.L. and **Keene, J.D.** (1982) RNA polymerase associated interactions near template promoter sequences of defective interfering particles of vesicular stomatitis virus. Journal of Virology 43: 241-249.

Schmidt, E.V., **Keene, J.D.**, Linial, M. and Smith, R.E. (1982) Association of 3' terminal RNA sequences with avian leukosis viruses causing a high incidence of osteopetrosis. Virology 116: 163-180.

Gaillard, R., Li, J.K.-K., **Keene, J.D.** and Joklik, W.K. (1982) The sequences of the termini of four genes of the three reovirus serotypes. Virology 121: 320-326.

Curriculum Vitae: Jack D. Keene

Kurilla, M.G., Piwnica-Worms, H. and **Keene, J.D.** (1982) Rapid and transient localization of the leader RNA of vesicular stomatitis virus in the nuclei of infected cells. Proceedings of the National Academy of Sciences (USA) 79: 5240-5244.

Piwnica-Worms, H. and **Keene, J.D.** (1983) Sequential appearance of short, capped vesicular stomatitis virus RNA transcripts in vitro. Virology 125: 206-218.

Kurilla, M.G. and **Keene, J.D.** (1983) The leader RNA of vesicular stomatitis virus is bound by a cellular protein reactive with anti-La lupus antibodies. Cell 34: 837-845.

Wilusz, J., Kurilla, M.G. and **Keene, J.D.** (1983) A host protein (La) binds to a unique species of minus sense leader RNA during replication of vesicular stomatitis virus. Proceedings of the National Academy of Sciences (USA) 80: 5827-5831.

Chambers, J.C., Kurilla, M.G. and **Keene, J.D.** (1983) Association between the 7S RNA and the lupus La protein varies among cell types. Journal of Biological Chemistry 258: 11438-11441.

Keene, J.D., Kurilla, M.G., Wilusz, J. and Chambers, J.C. (1984) *Interactions between cellular La protein and leader RNAs*. In: Nonsegmented Negative Strand Viruses, D.H.L. Bishop and R.W. Compans, eds., Academic Press, Inc., pp. 103-108.

Meier, E., Harmison, G.G., **Keene, J.D.** and Schubert, M. (1984) *Recombinational events during the generation of defective interfering particle RNAs of vesicular stomatitis virus*. In: Nonsegmented Negative Strand Viruses, D.H.L. Bishop and R.W. Compans, eds., Academic Press, Inc.

Kurilla, M.G., Holloway, B., Cabradilla, C. and **Keene, J.D.** (1984) Nucleotide sequence and host La protein interactions of the leader RNA of rabies virus. Journal of Virology 50: 773-778.

Wilusz, J. and **Keene, J.D.** (1984) Interactions of plus and minus strand leader RNAs of the New Jersey serotype of vesicular stomatitis virus with the cellular La protein. Virology 135: 65-73.

Meier, E., Harmison, G.G., **Keene, J.D.** and Schubert, M. (1984) The sites of copy choice replication involved in the generation of DI particle RNAs of vesicular stomatitis virus. Journal of Virology 51: 515-521.

Piwnica-Worms, H. and **Keene, J.D.** (1984) *Effects of cell extracts on transcription by virion and intracellular nucleocapsids of vesicular stomatitis virus*. In: Nonsegmented Negative Strand Viruses, D.H.L. Bishop and R.W. Compans, eds., Academic Press, Inc., pp. 109-114.

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Wilusz, J., Youngner, J.S. and **Keene, J.D.** (1985) Base mutations in the terminal non-coding regions of the genome of vesicular stomatitis virus isolated from persistent infections of L cells. Virology 140: 249-256.

Chambers, J.C. and **Keene, J.D.** (1985) Isolation and analysis of cDNA clones expressing human lupus La antigen. Proceedings of the National Academy of Sciences (USA) 82: 2115-2119.

Kole, R., Fresco, L.D., Cohen, P.L., Andrews, P.G. and **Keene, J.D.** (1985) Alu RNA-protein complexes found in vitro react with a novel lupus autoantibody. Journal of Biological Chemistry 260: 11781-11786.

Pisetsky, D.S., Hoch, S.O., Klatt, C., O'Donnell, M. and **Keene, J.D.** (1985) Specificity and idiotypic analysis of a monoclonal anti-Sm antibody with anti-DNA activity. Journal of Immunology 135: 4080-4085.

Piwnica-Worms, H. and **Keene, J.D.** (1985) Replication of vesicular stomatitis virus in permissive and nonpermissive cells. Journal of Biological Chemistry 260: 10503-10511.

Kurilla, M.G., Stone, H.O. and **Keene, J.D.** (1985) RNA sequence and transcriptional properties of the 3' end of the genome of Newcastle disease virus. Virology 145: 203-212.

Keene, J.D. (1985) Interactions between small viral RNAs of vesicular stomatitis virus and components of cellular gene expression. Microbiological Sciences 2: 152-156.

Wilusz, J. and **Keene, J.D.** (1986) Autoantibodies specific for U₁ RNA and initiator methionine transfer RNA. Journal of Biological Chemistry 261: 5467-5472.

Kiley, M.P., Wilusz, J., McCormick, J.B. and **Keene, J.D.** (1986) Conservation of the 3' terminal nucleotide sequences of the genomic RNA of Ebola and Marburg virus. Virology 149: 251-254.

Keene, J.D., Chambers, J.C. and Martin, B. (1987) *Interactions between the mammalian cell La protein and small RNAs.* In: DNA: Protein Interactions and Gene Regulation, E.B. Thompson and J. Papaconstantinou, eds., The University of Texas Press, Galveston, Texas.

Fresco, L.D., Kurilla, M.G. and **Keene, J.D.** (1987) Rapid inhibition of the processing and assembly of small nuclear ribonucleoproteins following infection with vesicular stomatitis virus. Molecular and Cellular Biology 7: 1148-1155.

Query, C.C. and **Keene, J.D.** (1987) A human autoimmune protein associated with U₁ RNA contains a region of homology that is immunologically cross-reactive with a retroviral group-specific antigen (P30gag). Cell 51: 211-220.

Curriculum Vitae: Jack D. Keene

Keene, J.D., Deutscher, S.L., Kenan, D. and Kelekar, A. (1987) Minireview: Nature of the La and Ro RNPs. Molecular Biology Reports 12: 235-238.

Deutscher, S.D. and **Keene, J.D.** (1988) A nucleic acid-specific conformational epitope on U1 RNA is recognized by a unique autoantibody. Proceedings of the National Academy of Sciences (USA) 85: 3299-3303.

Chambers, J.C., Martin, B.J.. Kenan, D.J. and **Keene, J.D.** (1988) Genomic structure and amino acid sequence domains of the human La autoantigen. Journal of Biological Chemistry 263: 18043-18051.

St. Clair, E.W., Pisetsky, D.S., Reich, C.F., Chambers, J.C. and **Keene, J.D.** (1988) Quantitative immunoassay of anti-La antibodies using purified recombinant La antigen. Arthritis and Rheumatism 31: 506-514.

St. Clair, E.W., Pisetsky, D.S., Reich, C.F. and **Keene, J.D.** (1988) Quantitative assessment of autoantibody binding to different regions of the La antigen expressed in recombinant fusion proteins. Journal of Immunology 141: 4173-4180.

Deutscher, S.D., Harley, J.B. and **Keene, J.D.** (1988) Molecular analysis of the 60kD human Ro ribonucleoprotein. Proceedings of the National Academy of Sciences (USA) 85: 9479-9483.

Query, C.C., Bentley, R.C. and **Keene, J.D.** (1989) A common RNA-recognition motif identified within a defined U1 RNA-binding domain of the 70K U1 snRNP protein. Cell 57: 89-101.

Lutz-Freyermuth, C. and **Keene, J.D.** (1989) The U1 RNA binding site of the U1 snRNP-associated A protein suggests a similarity with U2 snRNPs. Molecular and Cellular Biology 9: 2975-2982.

St. Clair, E.W., Talal, N., Moutsopoulos, H.M., Ballester, A., Zerva, L., **Keene, J.D.** and Pisetsky, D.S. (1989) Epitope specificity of anti-La antibodies from patients with Sjogren's syndrome. Journal of Autoimmunity 2: 335-344.

Frielle, D., Kim, P. and **Keene, J.D.** (1989) Inhibitory effects of vesicular stomatitis virus on cellular and influenza viral RNA metabolism and protein synthesis. Virology 172: 274-284.

St. Clair, E.W., Query, C.C., Bentley, R.C., **Keene, J.D.**, Polisson, R.P., Allen, N.B. and Pisetsky, D.P. (1989) Expression of autoantibodies to recombinant (U1) RNP-associated 70K antigen in systemic lupus erythematosus. Clinical Immunology and Immunopathology 54: 2934-2949.

Curriculum Vitae: Jack D. Keene

Keene, J.D. (1989) Molecular structure of the La and Ro autoantigens and their use in autoimmune diagnostics. Journal of Autoimmunity 2: 329-334.

Crone, D.E. and **Keene, J.D.** (1989) Viral transcription is necessary and sufficient for vesicular stomatitis virus to inhibit the maturation of small nuclear ribonucleoproteins. Journal of Virology 63: 4172-4180.

Query, C.C., Bentley, R.C. and **Keene, J.D.** (1989) A specific 31 nucleotide domain of U1 RNA directly interacts with the 70K U1 small nuclear ribonucleoprotein component. Molecular and Cellular Biology 9: 4872-4881.

Lutz-Freyermuth, C., Query, C.C. and **Keene, J.D.** (1990) Quantitative determination that only one of two potential RNA binding domains of the A protein component of the U1 small nuclear ribonucleoprotein complex binds with high affinity to stem-loop II of U1 RNA. Proceedings of the National Academy of Sciences (USA) 87: 6393-6397.

James, J.A., Dickey, W.D., Fujisaku, A., O'Brien, C.A., Deutscher, S.L., **Keene, J.D.** and Harley, J.B. (1990) Antigenicity of a recombinant Ro/SSA fusion protein. Arthritis and Rheumatism 33: 102-106.

St. Clair, E.W., Kenan, D., **Keene, J.D.** and Pisetsky, D.S. (1990) The fine specificity of anti-La antibodies induced in mice by immunization with recombinant human La autoantigen. Journal of Immunology 144: 3868-3876.

Sunde, L., Kjeldsen, E., Andoh, T., **Keene, J.D.** and Bolund, L. (1990) A three allele Taq 1 polymorphism at TOP1 gene. Nucleic Acids Research 18: 5919.

St. Clair, E.W., Burch, J.A., Ward, M.M., **Keene, J.D.** and Pisetsky, D.S. (1990) Temporal correlation of antibody responses to different epitopes of the human La autoantigen. Journal of Clinical Investigation 85: 515-521.

Tamura, H.-O., Kohchi, C., Yamada, R., Ikeda, T., Koiwai, O., Patterson, E., **Keene, J.D.**, Okada, K., Kjeldsen, E., Nishikawa, K. and Andoh, T. (1991) Molecular cloning of a cDNA of a camptothecin-resistant human DNA topoisomerase I and identification of mutation sites. Nucleic Acids Research 19: 69-75.

Keene, J.D. and Query, C.C. (1991) A Review: *Nuclear RNA Binding Proteins*. In: Progress in Nucleic Acid Research and Molecular Biology 41, W.E. Cohn and K. Moldave, eds., Academic Press, Inc., Orlando. pp. 179-202.

Fresco, L.D., Harper, D. and **Keene, J.D.** (1991) Leucine periodicity of A' protein implicated in U2 snRNP protein-protein interactions, Molecular and Cellular Biology 11: 1578-1589.

Hoffman, D.W., Query, C.C., Golden, B.L., White, S.W. and **Keene, J.D.** (1991) RNA binding domain of the A protein component of the U1 small nuclear ribonucleoprotein analyzed

Curriculum Vitae: Jack D. Keene

by NMR spectroscopy is structurally similar to ribosomal proteins. Proceedings of the National Academy of Sciences (USA) 88: 2495-2499.

St. Clair, E.W., Kenan, D., Burch, J.A., **Keene, J.D.** and Pisetsky, D.S. (1991) Anti-La antibody production by MRL-lpr/lpr: Analysis of fine specificity. Journal of Immunology 146: 1885-1892.

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*****Isolation of the first recombinant autoimmune antigens:***

- Demonstration of autoimmune epitopes and direct immune presentation**
- Methods to detect the presence of autoantibodies in patient sera**

Chambers, J.C. and **Keene, J.D.** (1985) Isolation and analysis of cDNA clones expressing human lupus La antigen. Proceedings of the National Academy of Sciences (USA) 82: 2115-2119.

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*****Discovery of conformational RNA epitopes and RNA-protein molecular mimicry:***

- Finding of crossreactivity of RNA and protein, and their structural mimicry***
- First demonstration of an aptamer blocking a protein-protein interaction***
- Hypothesis of an RNA-protein signaling network based on macromolecular mimicry***

Wilusz, J. and **Keene, J.D.** (1986) Autoantibodies specific for U₁ RNA and initiator methionine transfer RNA. Journal of Biological Chemistry 261: 5467-5472.

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-Discovery of the RRM (RNA Recognition Motif)

-Elucidation of a conserved RRM Superfamily (7th largest in human genome)

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*****Functional role for ELAV/Hu RRM proteins in neuronal plasticity and differentiation
- Translational activation and stabilization of early response gene messenger RNAs***

Levine, T.D., Gao, F., Andrews, L., King, P.H., and **Keene, J.D.** (1993) Hel-N1: an autoimmune protein with binding specificity for uridylate-rich 3' untranslated regions of growth factor messenger RNAs. Molecular and Cellular Biology 13: 3494-3504.

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****Development of "ribonomics" for parallel analysis of cellular mRNP infrastructure**

- Discovery of mRNA networks mediated by multi-targeted RNA-binding proteins**
- Discovery of clusters of structurally and functionally-linked mRNA subsets**
- Hypothesis for Post-Transcriptional Operons and Regulons in eukaryotes**

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A Comparative Review of Colony-Stimulating Factors

John Nemunaitis

Physician Reliance Network, Inc., and Sammons Cancer Center at Baylor University Medical Center, Dallas, Texas, USA

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Summary

The efficacy of dose-intensive chemotherapy in oncology is limited by the duration and severity of neutropenia. Several recombinant DNA factors that alter neutrophil proliferation and function, and are characterised by their ability to stimulate colony formation of myeloid progenitors *in vitro*, have been shown to alter clinical sequelae associated with neutropenia *in vivo*. Two of these factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been approved by the US FDA. One other factor, macrophage colony-stimulating factor (M-CSF), is approved as indicated therapy in Japan. The clinical effects of these agents are compared in this review. Results of clinical trials suggest that the efficacy of G-CSF is greatest when used as an agent to enhance circulation of stem cells and pre-colony-forming progenitor cells. It is also an effective agent in reducing the duration of neutropenia following dose-intensive chemotherapy, thereby leading to a reduction in the incidence of febrile neutropenia. Similar observations were made with GM-CSF, although toxicity with the latter agent appears to be moderately greater than that observed with G-CSF. Functional activity of GM-CSF is broader than that of G-CSF, in that macrophages are affected by GM-CSF. As a result, some

data suggest that GM-CSF may be more applicable to patients with a high risk of infection. There is a suggestion that M-CSF assists neutrophil recovery, although this effect may be indirect, via the induction of other cytokines. The predominant effect of M-CSF appears to be enhancement of macrophage and monocyte function, which may reduce the severity and duration of fungal infection.

Three distinct recombinant human (rh) growth regulatory factors, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF), which influence functional activity, survival, proliferation and differentiation of myeloid haemopoietic cells, have been identified and molecularly cloned. Each has been approved worldwide for clinical use (to date, M-CSF only in Japan). The activity of G-CSF focuses on proliferation, functional stimulation and differentiation of committed progenitors of neutrophils. GM-CSF has activity similar to that of G-CSF, but is directed towards an earlier progenitor population capable of differentiation towards a monocyte, neutrophil or granulocyte lineage. Both G-CSF and GM-CSF stimulate mobilisation of multipotent progenitors and stem cells from marrow to circulation. The activity of M-CSF focuses on proliferation, functional enhancement and differentiation of monocytes and macrophages.

Preclinical studies indicate improved survival in animal models tested with each cytokine when administered prophylactically before or after cytotoxic chemotherapy. Survival has also been shown to be improved in nonmyelosuppressed animal models given prophylactic G-CSF, M-CSF or GM-CSF before the introduction of bacterial infection and with GM-CSF and M-CSF before the introduction of fungal infection with a variety of *Candida* species. M-CSF has also been shown to improve survival in 1 animal model when administered after the establishment of fungal infection (renal and hepatic abscess).

Evidence from preclinical work suggests not only potential clinical efficacy with use of these cytokines in neutropenia, but also a possible application in patient populations at risk of, or with,

active infection. The purpose of this review is to identify established and potential new clinical applications of G-CSF, GM-CSF and M-CSF.

1. Granulocyte Colony-Stimulating Factor

The G-CSF filgrastim is indicated therapy in the US as prophylaxis following myelosuppressive chemotherapy, bone marrow transplant (BMT) and severe chronic neutropenia, and for mobilisation of peripheral blood progenitor cells. Other formulations of G-CSF (i.e. lenograstim) with similar activity are also approved worldwide.

The initial phase III trial investigating G-CSF was done in 210 patients with small-cell lung cancer receiving cyclophosphamide, doxorubicin and etoposide.^[1] G-CSF was administered subcutaneously at a dosage of 4 to 8 µg/kg/day from days 4 to 17 after completion of chemotherapy. The duration of neutropenia was markedly shortened in patients receiving the agent during cycle 1 (from 5.6 to 2.4 days) and it was reduced from 3 days to 1 day over all cycles. Additionally, the incidence of febrile neutropenia was reduced (76% in placebo-treated patients, 40% in G-CSF-treated patients). Only 52% of G-CSF-treated patients were hospitalised as opposed to 69% of placebo-treated patients. The incidence of neutropenia was 57% (286 of 500 cycles) in the patients who received G-CSF compared with 77% (416 of 543 cycles) for patients randomised to placebo.

Areas of efficacy as suggested by this trial have been studied in numerous other controlled trials utilising myelosuppressive chemotherapy to treat patients with other solid tumours.^[1-30] Reduced duration of neutropenia was consistently observed in patients receiving G-CSF. No evidence of tumour stimulation was seen. Reduction

of febrile neutropenia was commonly observed,^[1,2,5-7,10,11,13,18,20,27,29] and a lower rate of documented infection^[1,9,15,18] and hospitalisation was recorded in some trials,^[1,6,15] but more commonly, the actual clinical benefit in patients receiving G-CSF was either not described or was not observed. Improvement in survival was only rarely observed.^[13]

G-CSF was well tolerated in these trials. Toxicity described in the initial phase III trial revealed mild to moderate medullary pain in 24% of patients, which was generally controlled with non-narcotic analgesics.^[11] Itching and rashes were also reported to have a higher frequency than in placebo-treated patients. Other studies have described febrile episodes, bone pain and abdominal pain to be more frequent in patients receiving G-CSF in doses above 8 µg/kg/day.^[1-30] Rare adverse reactions have included reversible elevations in uric acid, lactic dehydrogenase and alkaline phosphatase, seizures, anaphylactic reactions and transient decreases in blood pressure.

Efforts to utilise G-CSF to escalate myelosuppressive dose levels of cytotoxic chemotherapy have been extensive (see table I).^[31-65] Leukopenia continues to be the dose-limiting toxicity of most myelosuppressive regimens evaluated. Higher than expected response rates have been reported, and survival duration is often improved in comparison with previously published survival rates using standard dosage regimens, prompting suggestions of potential benefit obtained from the use of G-CSF and dose-intensive therapy.

Unfortunately, of the 35 trials reviewed in table I, none evaluated dose intensity in phase III trials measuring survival. Therefore, no conclusions can be drawn confirming the efficacy of dose-intensive approaches using prophylactic G-CSF compared with standard chemotherapy or other dose-intensive approaches using prophylactic oral antibiotics to reduce neutropenia-related complications. As a consequence, many practitioners do not consider using G-CSF until after the occurrence of a febrile episode in an early cycle of a multicycle chemotherapy regimen. At this point,

the use of G-CSF is recommended to maintain the planned dose intensity.

Maher et al.^[66] and Mayordomo and colleagues^[67] reviewed the effects of G-CSF, GM-CSF or placebo administered at the time of occurrence of febrile neutropenia. The results of these trials suggested only a limited value in initiating either cytokine after the observation of febrile neutropenia had been made: although neutropenia duration was shortened by 1 day with active treatment, there were no significant differences between active treatments and placebo in both fever duration and percentage of patient deaths after 4 weeks.

1.1 Bone Marrow Transplantation

Several trials have been performed confirming that patients who receive G-CSF achieve an absolute neutrophil count (ANC) of $\geq 500/\text{mm}^3$ earlier than controls^[68-85] following autologous or allogeneic BMT. Neutrophil recovery to 500 cells/mm³ is generally 7 days earlier, platelet recovery is not affected, infection is either not affected or is less frequent, and hospital stay is generally not affected or is of shorter duration in G-CSF-treated patients. No adverse effects of G-CSF with graft-versus-host disease (GVHD), rate of relapse, survival, or the occurrence of graft failure or rejection have been observed. A daily subcutaneous route of administration between 5 and 10 µg/kg/day is well tolerated.

1.2 Peripheral Blood Progenitor Cell Transplant

The results of recent trials indicate that sufficient numbers of committed and multipotent progenitor cells can be harvested from the circulation following administration of G-CSF, and reinfused, to enhance neutrophil and platelet recovery after myeloablative or myelosuppressive chemo- or radiotherapy. Data from most trials indicate the need for a 6- or 7-day course of G-CSF. Peak circulation of progenitors occurs on days 4, 5 and 6. Infusion of G-CSF mobilised peripheral blood progenitor cells following dose-intensive (severe

Table I. Use of granulocyte colony-stimulating factor (G-CSF) to alter dose intensity of myelosuppressive regimens

Solid tumour malignancy	Regimen	No. of patients	Effect of G-CSF on dose intensity	Limiting toxicity	Reference
Breast	F, Ep, C	64	MTD Ep = 120 mg/m ² /day	Leucopenia	55
	F, Ep, C	14	F, Ep, C q2wk	Thrombocytopenia	54
	F, Ep, C	32	CEpF q2wk x 6 (93% of patients)	No grade 4	50
	T	25	T = 250 mg/m ² /day (second-line)	Leucopenia	41
	T	52	T = 200 mg/m ² /day (third-line)	Leucopenia	41
	Mi, F.	22	MTD Mi = 24 mg/m ² /day	Leucopenia	51
	Ep	50	Ep = 110 mg/m ² q2wk	Stomatitis	40
	I, Ep	20	I, Ep q2wk	Leucopenia	53
	Ep	42	EP = 110 mg/m ² /day (every 4wk)	Leucopenia	40
	Mi, N	43	MTD Mi = 6 mg/m ² /wk, N = 30 mg/m ² /wk	Leucopenia	56
	I, A	18	MTD I = 2.75g/m ² x 5 days	Thrombocytopenia	39
	C, A, F	37	MTD C = 4000 mg/m ² , A = 120 mg/m ²	Leucopenia	35
	Mi, M, My	24	MTD Mi = 12 mg/m ² /day	Thrombocytopenia, lethargy	38
	C, Ep, F	30	MTD Ep = 40 mg/m ² /day	Leucopenia	37
Hodgkin's disease	CEAVP	22	MTD C = 1500 mg/m ² /day, E = 160 mg/m ² /day	Leucopenia	36
NHL	CAOP	27	MTD C = 1500 mg/m ²	Leucopenia	64
	COP-BLAM	72	3wk → 2wk schedule	Neutropenia	31
Non-small-cell lung	Cb, E (oral)	39	MTD Cb = AUC 8	Thrombocytopenia	65
	N, Cb	22	MTD Cb AUC 7/4wk, N = 30 mg/m ² /wk	Leucopenia	46
	N, Ep	18	MTD Ep = 90 mg/m ² /day	Leucopenia	52
	Ir, Ci	20	MTD Ir = 80 mg/m ² on days 1,8,15	Diarrhoea	58
Ovarian	Cb	21	MTD Cb = AUC 9 every 2wk	Thrombocytopenia	44
	T	14	MTD T = 300 mg/m ² /day	Peripheral neuropathy	33
	T	47	T = 250 mg/m ² /day	Leucopenia	42
Ovarian/breast	A	17	MTD A = 375 mg/m ² over 6wk	Mucositis	32
Small-cell lung	V, Ci	46	MTD VM-26 = 100 mg/m ² /day × 5 days	Thrombocytopenia	48
	T	37	T = 250 mg/m ² /day	Leucopenia	45
	Ci,E - I,A	40	Alternating weeks maintained in 82%	Leucopenia	57
	V, Ci	13	MTD V = 120 mg/m ² × 3 days	Thrombocytopenia	34
Testicular	BLEOP	13	Reduced treatment delays	Thrombocytopenia	49
Urothelial	M-Vb, A, Ci	35	25% dose increase	Early death	59
Various	T, Ci	32	MTD T = 250 mg/m ² , Ci = 75 mg/m ²	Neurotoxicity	63
	Ir, E	33	MTD Ir = 60 mg/m ² on days 1-3, E = 60 mg/m ² on days 1-3	Diarrhoea	62
	T (3 hr)	35	MTD T = 300 mg/m ²	Peripheral neuropathy	47
	T, To	46	MTD T = 230 mg/m ² /day	Neuromuscular	43
	Ci, To	38	MTD C = 75 mg/m ² /day, To = 1 mg/m ² /day × 5 days	Leucopenia	60
	Pi	38	MTD Pi = 185 mg/m ²	Leucopenia	61

Abbreviations: A = doxorubicin (adriamycin); AUC = area under the concentration-time curve; BL = bleomycin; C = cyclophosphamide; Cb = carboplatin; Ci = cisplatin; E = etoposide; Ep = epirubicin; F = fluorouracil; I = ifosfamide; Ir = irinotecan; M = methotrexate; Mi = mitoxantrone; MTD = maximum tolerated dose; My = mitomycin; N = vinorelbine; NHL = non-Hodgkin's lymphoma; O = vincristine; q2wk = every 2 weeks; P = prednisone; Pi = piroxantrone; T = paclitaxel; To = topotecan; V = teniposide (VM-26); Vb = vinblastine.

myelosuppressive) chemo- or radiotherapy, leading to a decrease in the number of days required for neutrophil and platelet recovery, and reductions in

hospital stay.^[86-104] Results of G-CSF-mobilised peripheral blood stem (progenitor) cell (PBSC) infusion following myeloablative chemotherapy are

summarised in table II.^[105-120] These results suggest that neutrophil and platelet recovery is more rapid in patients who receive PBSC mobilised with G-CSF compared with those who received BMT, regardless of whether G-CSF was administered after BMT.

A reduction in the number of episodes of febrile neutropenia, the number of platelet transfusions and duration of hospital stay has been observed in some studies.^[105,107,108,112,116,120] The optimal dosage of G-CSF for mobilisation is 10 µg/kg/day, administered as a single daily subcutaneous injection. G-CSF can also be administered for mobilisation following modest cytotoxic chemotherapy. Enhanced concentration of circulating progenitor cells is observed; however, it remains unclear if the added toxicity related to the cytotoxic agent used for mobilisation improves clinical outcome. Long term engraftment has been found to be stable in patients who have received peripheral blood progenitor cells. The use of allogeneic PBSCs mobilised by G-CSF is in early investigation, but results suggest more rapid neutrophil and platelet recovery compared with allogeneic marrow infusion and no effect on acute GVHD, although chronic GVHD may be increased.^[121-125]

1.3 Chronic Neutropenia

Patients with idiopathic chronic neutropenia or congenital neutropenia experience increased morbidity and mortality as a result of recurrent infection related to the neutropenic state. Administration of G-CSF significantly increases and sustains a higher neutrophil level, resulting in less infection and hospital time.

In one trial^[126] involving 123 patients (median age 12 years) with severe chronic neutropenia ($\text{ANC} < 500/\text{mm}^3$), G-CSF was administered daily to 1 group of patients by subcutaneous injection with dose adjustments to maintain an ANC of $> 1500/\text{mm}^3$; another group received no therapy for 4 months. After 4 months, a crossover of patients who did not receive G-CSF was allowed. All patients ended up receiving G-CSF. 108 patients

achieved a median ANC of $\geq 1500/\text{mm}^3$ while receiving G-CSF.

The incidence and duration of infection was reduced 50%, the incidence of oral pharyngeal ulcers was reduced from 26 to 0%, and antibiotic use was reduced from 49 to 20% while patients were receiving G-CSF. 28 hospitalisations occurred in patients receiving G-CSF compared with 44 in patients not receiving the drug over the same period. The median ANC was $210/\text{mm}^3$ in patients who did not receive G-CSF, and it was maintained above $1500/\text{mm}^3$ in patients who did. Patients with congenital neutropenia appeared to require higher dosages of G-CSF (2.2 to 4 µg/kg/day) than patients with idiopathic or cyclic neutropenia (0.5 µg/kg/day) in order to achieve an ANC of $\geq 1500/\text{mm}^3$. Mild to moderate bone pain was reported in 30 to 40% of patients and was controlled with non-narcotic analgesics. Splenomegaly developed in 30% of patients after treatment with G-CSF; 6% developed thrombocytopenia (platelet count $< 50\,000/\text{mm}^3$). This appeared to correlate with the onset of splenomegaly. Myelodysplasia or leukaemia developed in 3% of patients, and 12% who had normal cytogenetic studies at baseline were found to have abnormalities 18 to 52 months after initiation of G-CSF.

Since acute leukaemia and myelodysplastic syndrome may occasionally be preceded by a state of severe neutropenia, it is difficult to determine if this rare occurrence of leukaemia is related to G-CSF or is part of the natural history of disease.^[127-129] Results of other phase I/II trials have also been consistent with results observed in this trial;^[127,128] therefore, since quality of life is improved,^[130] G-CSF is recommended for prophylactic use in patients with severe chronic neutropenia ($\text{ANC} < 500/\text{mm}^3$).

1.4 Leukaemia and Leukaemia-Related Syndromes

G-CSF stimulates the proliferation of myeloid leukaemia blasts^[131] *in vitro*, leading to concerns with clinical use in acute leukaemia. However, controlled trials have failed to supply any evidence

Table II. Results of granulocyte colony-stimulating factor (G-CSF)-mobilised peripheral blood stem (progenitor) cell infusion (PBSC) following myeloablative chemotherapy

Solid tumour malignancy	Mobilising regimen	No. of patients	Day when ANC > 500/mm ³		Day when platelet count > 50 000/mm ³		Duration of hospital stay (days)		Cytokine postinfusion		Reference	
			BMT		PBSC		BMT		BMT			
			BMT	PBSC	BMT	PBSC	BMT	PBSC	BMT	PBSC		
Breast	F, Ep, C/G-CSF	29	9								109	
Breast, non-Hodgkin's lymphoma	G-CSF	15 ^a	19	13		35	27	18	+	+	105	
	IL-3 → G-CSF	23 ^a	19	12		25	27	19	+	+	105	
Hodgkin's disease, non-Hodgkin's lymphoma	Chemo/G-CSF	10		15					-	-	114	
Multiple myeloma	C, P/G-CSF	37		12					+	+	110	
Neuroblastoma (paediatric)	C, E/G-CSF	5	18						-	-	115	
	G-CSF	6		14					-	-	115	
Non-Hodgkin's lymphoma	G-CSF	26	10						+	+	118	
	G-CSF	27 ^b	14	11		23	17	+	+	+	116	
	CA, M/G-CSF	30	13						-	-	117	
	G-CSF	39	10						+	+	118	
	CA, M/G-CSF	20	12			16.5	21	-	-	-	119	
	CA, M/G-CSF	20	10			14.5	21	-	+	+	119	
	G-CSF	29	10			15	13	-	+	+	111	
Non-Hodgkin's lymphoma, breast	G-CSF	49 ^a	19	10					-	-	112	
Various	CA, E/G-CSF	42 ^a		13					-	-	120	
	C/G-CSF	42		14					-	-	113	
	G-CSF	12		13					-	-	106	
	G-CSF	14 ^a	10	9	39	15	17	14	+	+	107	
	G-CSF	34 ^a	21	15	31	18	31	22	+	+	108	

^a Historical BMT controls (the prospective patients received the cytokine).^b Prospective BMT controls.

Abbreviations: A = Doxorubicin (adriamycin); ANC = absolute neutrophil count; BMT = bone marrow transplant; C = cyclophosphamide; CA = cytarabine; Chemo = chemotherapy; E = etoposide; Ep = epirubicin; IL = interleukin; Ml = mitoxantrone; P = prednisone; - = cytokine not administered; + = cytokine administered.

that G-CSF adversely affects the time of leukaemia relapse, response rate, duration of response or survival.^[132-140] Neutrophil recovery has occurred at an earlier rate in patients receiving G-CSF following induction or consolidation chemotherapy. Platelet recovery and infection have not been affected, although duration of hospital stay was reduced in only 1 trial^[135] despite improvements in ANC in all trials, except one in which ANC was not reported.^[138]

In one trial,^[141] G-CSF was administered to neutropenic leukaemia patients at the onset of documented sepsis in one group of patients ($n = 16$ episodes), and comparison was made with patients being treated in the same manner, but without G-CSF. There was no statistical difference in mortality related to sepsis between these groups.

G-CSF has also been administered to patients with refractory anaemia and myelodysplastic syndrome.^[142-147] It was well tolerated and neutrophil levels increased without adverse effects on blast cells. Platelet counts were not affected. To date, no trials have been published looking at the effect of the prophylactic use of G-CSF on infection in myelodysplastic syndrome.

One trial comparing G-CSF with placebo found shorter survival in patients with refractory anaemia and excess blasts, although it is unclear if the survival difference was related to G-CSF or prognostic characteristics between the two groups.^[146] Recently, it was observed that patients with myelodysplastic syndrome who received the combination of G-CSF and erythropoietin had a greater elevation of haemoglobin levels than when erythropoietin alone was given.^[147]

G-CSF has also been given in combination with antithymocyte globulin (ATG) and cyclosporin to aplastic anaemia patients with severe neutropenia ($<500/\text{mm}^3$).^[145] In one trial involving 40 patients, it was well tolerated; 33 patients responded to ATG with trilineage engraftment and became transfusion-independent a median of 115 days after initiation of treatment.

2. Granulocyte-Macrophage Colony-Stimulating Factor

In the US, GM-CSF derived from yeast is indicated as therapy in neutropenic patients after autologous or allogeneic BMT, and for mobilisation of autologous peripheral blood progenitor cells. *Escherichia coli*-derived GM-CSF is also approved in Europe for prophylactic treatment following dose-intensive chemotherapy.

The recommended dose of GM-CSF is 250 µg/m²/day administered daily as a 2- or 4-hour intravenous infusion, although activation and tolerability using the same dosage and schedule, but administered subcutaneously, is not different. GM-CSF administration is contraindicated in patients with excessive myeloid leukaemia blasts (>10%) in the bone marrow or peripheral blood, and during concomitant administration with radiotherapy or chemotherapy.

The toxicity attributed to this agent in healthy volunteers includes low-grade fevers, abdominal/bone pain, fluid retention, headaches and transient rashes in 10 to 30% of patients. These toxicities are difficult to identify in prospective, controlled trials involving dose-intensive chemotherapy, since they also occur as a natural consequence of the treatment regimen.

2.1 Myelosuppressive Chemotherapy

GM-CSF has similar activity to G-CSF in the treatment of patients receiving myelosuppressive chemotherapy, but toxicity (low grade fevers, myalgias, bone pains, abdominal pains) is considered slightly greater. The US FDA has not approved the use of GM-CSF for solid tumour patients receiving myelosuppressive chemotherapy. Results of trials with GM-CSF in patients receiving myelosuppressive chemotherapy reported a reduction in neutropenia,^[148-153] while reported reductions in infection and hospital stay were reported additionally in some studies.^[152,153] Further work exploring the use of this agent with dose-intense regimens is ongoing.

2.2 Autologous Bone Marrow Transplant

The primary trial in autologous BMT which led to approval of GM-CSF in the US was a multi-centre trial involving patients with non-Hodgkin's lymphoma and acute lymphocytic leukaemia.^[154] In this trial, time to achieve an ANC of $>500/\text{mm}^3$ was 6 days shorter (18 vs 24 days) and that taken to reach a figure of $\geq 1000/\text{mm}^3$ was 8 days shorter (24 vs 32 days), and the duration of hospitalisation was 10 days less (21 vs 31 days) in patients receiving GM-CSF, compared with placebo recipients. Duration of infection and duration of antibacterial therapy were also significantly shorter in GM-CSF-treated patients. Additional trials have confirmed the results of the initial phase III study with rhGM-CSF.^[154-165] The incidence and type of adverse effects in patients receiving either GM-CSF or placebo were not statistically significantly different.

In one retrospective analysis, infectious complications in 106 consecutive historical patients who underwent autologous BMT for lymphoid malignancy were compared with those in 50 consecutive, similarly treated patients who received prophylactic GM-CSF (Nemunaitis J, et al., unpublished data). 40% of control patients developed documented infection compared with only 13% of the GM-CSF-treated patients. It was suggested that there was a benefit from GM-CSF during the period of severe neutropenia before differences in neutrophil levels between the study groups were detectable, suggesting evidence to support the use of GM-CSF for its functional effects.

2.3 Marrow Graft Failure

Nearly 1% of HLA-matched sibling allogeneic donor transplant patients, 5% of unrelated donor transplant patients, and 10 to 15% of mismatched allogeneic transplant patients will have delayed neutrophil recovery resulting from immunological rejection of donor cells.^[166] Graft failure without evidence of immunological rejection can also occur. Potential causes of nonimmunological graft failure include low stem cell inoculum, post-

transplant infection (i.e. cytomegalovirus) or drug toxicity. With the exception of patients with aplastic anaemia, fewer than 20% of patients not treated with GM-CSF will survive 5 years.^[167,168]

For the purpose of evaluating the use of GM-CSF in the setting of marrow graft failure, a uniform definition of graft failure was adopted in one large trial.^[167] Patients who did not achieve a neutrophil level of $>100/\text{mm}^3$ by day 28 after transplant, those who did not achieve a neutrophil count of $100/\text{mm}^3$ by day 21 after transplant with evidence of infection, and those who initially achieved an ANC of $>500/\text{mm}^3$ for at least 1 week and who subsequently dropped to $<500/\text{mm}^3$ for at least 1 week, were defined as having graft failure. Historical patients who fulfil this definition have a 2-year survival of less than 20%.^[167]

The predominant cause of death of patients with graft failure is infection. In an initial trial, GM-CSF was administered at a dose of $250 \mu\text{g}/\text{m}^2/\text{day}$ by 2-hour intravenous infusion for 14 days. If the neutrophil count did not reach $>500/\text{mm}^3$ within 3 weeks of therapy, a second, and possibly a third, course of GM-CSF was administered.^[167,169]

The drug was well tolerated in an initial phase I/II trial^[167] and was, therefore, explored in a more extended study involving 185 patients.^[169] The median survival of patients undergoing allogeneic BMT who received GM-CSF was 97 days, compared with 35 days in a historical matched control group.^[160,170] In the case of autologous BMT, the figure was 474 days, against 161 days in a historical matched control group. Multivariate analysis of possible factors that may affect survival in patients receiving GM-CSF failed to identify patients more likely or less likely to respond. Improvement in survival and reduction of infection-related mortality was also observed in other trials exploring the use of GM-CSF in patients with marrow graft failure.^[171,172]

2.4 Allogeneic Bone Marrow Transplant

Given the evidence of efficacy with GM-CSF in autologous transplant, and lack of toxicity in both autologous and allogeneic transplant patients with

graft failure, phase I/II trials in patients undergoing matched sibling and unrelated donor transplant were performed (see table III).^[173-184] The results revealed earlier neutrophil recovery, occasional improvement in infection rates and shorter duration of hospitalisation. No adverse effects on GVHD or survival were observed. Phase III trials with GM-CSF in allogeneic transplant recipients confirmed the efficacy of the drug.

In the FDA-approval trial, the time to achieve a neutrophil level of 500/mm³ in GM-CSF-treated patients was 4 days shorter than that with placebo (13 vs 17), the time to achieve an ANC of $\geq 1000/\text{mm}^3$ was 5 days shorter (14 vs 19), the number of patients with infection was fewer (30 vs 42), the number of patients with bacteraemia was smaller (9 vs 19), and fewer days were spent in

hospital (24 vs 25). Interestingly, the incidence of severe mucositis (grade III/IV) was also significantly improved in the GM-CSF group (4 of 53 vs 16 of 56) compared with placebo in this trial; however, mucositis has not been shown to be affected in other trials. The severity or duration of GVHD, relapse rates and survival were not different between GM-CSF- and placebo-treated patients. Patients undergoing unrelated bone marrow transplant also showed earlier neutrophil recovery, but no other factors such as infection, hospital duration or mucositis were improved.^[175]

2.5 Peripheral Blood Stem Cell Transplant

The minimum number of mononuclear cells required for consistent engraftment rates is between

Table III. Granulocyte-macrophage colony-stimulating factor (GM-CSF) in allogeneic bone marrow transplant

Reference	Cytokine	Type of BMT	No. of patients	GVHD prophylaxis	Day when ANC > 500/mm ³	Day when patient platelet-independent	Percentage of GVHD \geq grade III ^a	Survival (y) [%]
Dewitte et al. ^[173]	Placebo	Matched sibling	28	T cell depletion	20	NR	6	2 [40]
	GM-CSF	Matched sibling	29	T cell depletion	15	NR	3	2 [58]
Powells et al. ^[174]	Placebo	Matched sibling	20	CSP	16	NR	15	1 [20]
	GM-CSF	Matched sibling	20	CSP	13	NR	5	1 [42]
Anasetti et al. ^[175]	Placebo	Unrelated	63	CSP/MTX	22	NR	NR	1 [51]
	GM-CSF	Unrelated	61	CSP/MTX	20	NR	NR	1 [39]
Nemunaitis et al. ^[176]	Placebo	Matched sibling	56	CSP/P	17	24	12	1 [63]
	GM-CSF	Matched sibling	53	CSP/P	13	20	15	1 [55]
Hiraoka et al. ^[177]	Placebo	Matched sibling	16	CSP/MTX	22	NR	NR	1 [56]
	GM-CSF	Matched sibling	16	CSP/MTX	14	NR	NR	1 [48]
Nemunaitis et al. ^[178]	Historical controls	Matched sibling	50	CSP/P	19	21	ND	
	GM-CSF	Matched sibling	28	CSP/P	14	23	14	
Nemunaitis et al. ^[179,180]	Historical controls	Matched sibling	43	CSP/MTX	24	20	ND	
	GM-CSF	Matched sibling	19	CSP/MTX	20	23	6	
	Historical controls	Unrelated	78	CSP/MTX	23	31	ND	2 [49]
	GM-CSF	Unrelated	103	CSP/MTX	21	23	25	2 [57]
Naparstek et al. ^[181]	Historical controls	Matched sibling	40	CSP/MTX	18	23	ND	
	GM-CSF	Matched sibling	20	CSP/MTX	14	16	ND	
Nemunaitis et al. ^[182]	GM-CSF	Unrelated	9	CSP/P	16	NR	50	
Chap et al. ^[183]	GM-CSF	Matched sibling	2	CSP/P	13	NR	50	
Nemunaitis et al. ^[184]	GM-CSF	Matched sibling	6	CSP/P	12	14	0	

a Grade III or IV GVHD indicates that the condition is 'very severe'.

Abbreviations: ANC = absolute neutrophil count; BMT = bone marrow transplant; CSP = cyclosporin; GVHD = graft-versus-host-disease; MTX = methotrexate; ND = not different from comparator group (specific percentages not reported); NR = not reported; P = prednisone.

Table IV. Morbidity related to mobilisation with cyclophosphamide at dosages shown

	Kotasek et al. ^[188]		To et al. ^[189]		Jagannath et al. ^[187]		Boliron et al. ^[188]		Rosenfeld et al. ^[190]		Sureda et al. ^[185]	
	7 g/m ²	4 g/m ²	7 g/m ²	4 g/m ²	6 g/m ²	6 g/m ² + GM-CSF	7 g/m ²	7 g/m ² + GM-CSF	4 g/m ²	4 g/m ² + GM-CSF	4 g/m ²	4 g/m ² + GM-CSF
No. of cycles	23	52	23	37	36	39	21	10	10	10	12	15
Day when ANC <1000/ μ m ³	10/NR	7/NR	NR/NR	NR/NR	NR/18	NR/15	NR/20	NR/14	15/NR	12/NR	NR/10	NR/7
Day when platelet count <50 000/ μ m ³	7	1	NR	NR	18	15	15	13	NR	NR	NR	NR
Percentage of patients with febrile neutropenia	100	21	100	44	57 ^a	57 ^a	NR	NR	50	0	92	73
Percentage of sepsis	39	10	NR	NR	23 ^a	23 ^a	5	20	NR	NR	42	26
Duration of hospital stay (days)	NR	NR	NR	NR	NR	NR	23	22	NR	NR	NR	NR

^a 57% of patients developed febrile neutropenia and 23% documented infections in both groups. GM-CSF was described as not making a difference in infection morbidity but data were not shown.

Abbreviations: ANC = absolute neutrophil count; GM-CSF = granulocyte-macrophage colony-stimulating factor; NR = not reported.

3×10^8 and 6×10^8 cells/kg.^[185] Primitive and committed progenitor cells express CD34 antigen. Levels of harvested CD34 surface antigen expressive cells have been shown to be predictive of the rate of neutrophil and/or platelet recovery after peripheral blood stem cell transplant (PBSCT). A minimum of 2×10^6 CD34+ cells/kg are necessary to achieve rapid, consistent and sustained engraftment.^[185]

Administration of cyclophosphamide (at a variety of doses ranging from 4 to 7 g/m²), the combination of GM-CSF with cyclophosphamide, and the administration of a variety of chemotherapy agents with or without GM-CSF, are other methods which have been shown to be effective strategies for mobilisation of progenitor cells. Each of the methods described has certain advantages and disadvantages.

Mobilisation with cyclophosphamide, when that drug is combined with rhGM-CSF, induces a greater volume of circulating progenitor cells than mobilisation by cytokines alone. However, the toxicity related to cyclophosphamide may be significant^[186-191] (see table IV). Morbidity related to the duration of pancytopenia, febrile neutropenia and infection has been associated with substantial hospitalisation and occasional mortality. In one trial, patients were kept in the hospital for 23 days after the administration of cyclophosphamide for mobilisation.^[189] In this trial, the addition of rhGM-CSF did not appear to reduce morbidity, despite an improvement in neutrophil recovery.

An advantage to mobilising PBSC with chemotherapy is that most patients requiring mobilisation often have progressive disease and may not be able to wait 1 to 2 weeks for mobilisation with cytokines alone before receiving antitumour agents. There is no significant evidence that tumour cells are mobilised into circulation during recovery after chemotherapy alone, after chemotherapy combined with growth factors or after growth factors alone. Contaminating tumour cells are less frequently identified in mobilised peripheral blood than in bone marrow.^[192-195] The rate of neutrophil recovery and the frequency of clinical complica-

Table V. Cytokine-mobilised peripheral blood stem-cell transplant versus historical bone marrow transplant, with and without cytokines^[204]

	Arm ^a			Historical placebo	Historical GM-CSF
	1	2	3		
Mononuclear cell count/kg/apheresis	2.5	1.3	1.3		
Day when absolute neutrophil count					
>100/mm ³	12	16	11	14	13
>500/mm ³	14	24	13	26	19
>1000/mm ³	16	23	15	33	26
Day when platelet count > 20 000/mm ³	15	28	10	29	26
Duration of hospital stay (days)	19	27	18	33	27

a Arm 1 = rhIL-3 5 µg/kg/day prior to rhG-CSF 5 µg/kg/day; arm 2 = rhIL-3 5 µg/kg/day prior to rhGM-CSF 5 µg/kg/day; arm 3 = rhIL-3 5 µg/kg/day combined with rhG-CSF 5 µg/kg/day.

Abbreviation: rhIL-3 = recombinant human interleukin-3.

tions following infusion of GM-CSF-mobilised PBSCs are similar to those obtained with G-CSF.^[187,190,196-202]

Over the past 2 years, methods of mobilising PBSCs have changed. Few centres now mobilise PBSCs following chemotherapy without cytokines. Most centres harvest the cells after mobilisation with chemotherapy combined with cytokines, or after cytokines alone. Nonrandomised studies performed in similar patient populations, receiving similar preparative regimens, reveal a reduction in duration of neutropenia and duration of hospital stay in patients receiving cytokines after marrow transplant, and further improvement in patients receiving PBSCT.^[203]

Studies directly comparing PBSCT against autologous BMT in similar patient populations suggest that there is a marked advantage to the use of PBSCs after myeloablative regimens over BMT with or without prophylactic cytokines, particularly with respect to platelet recovery.^[190,191] Rapid neutrophil and platelet recovery is important for maintenance of a dose-intensive chemotherapy regimen, and may reduce the cost of intensive therapy. Overall, despite the lack of completed randomised trials, data suggest that the use of stem cells and progenitor cells contained in peripheral blood substantially reduces morbidity compared with the results achieved with bone marrow transplant.

Other cytokines are also being explored to potentiate the effects of GM-CSF or G-CSF for mobi-

lisation. Table V compares engraftment rates in patients receiving IL-3/G-CSF- and IL-3/GM-CSF-mobilised cells with those in historical BMT patients who received no cytokines or GM-CSF after marrow infusion.^[204] Engraftment rates in patients receiving cytokine-mobilised PBSCs and in historical BMT recipients in table V are consistent with other published data.

2.6 Disorders in Marrow Function

Several phase II studies show increases in neutrophil levels in patients with aplastic anaemia and myelodysplastic syndrome who receive GM-CSF, although in patients with more severe aplastic anaemia (ANC <100/mm³) neutrophil stimulation is not as significant. Stimulation of non-neutrophil lineages and infection is not affected.^[205-208] Patients with other states of chronic neutropenia have also been investigated using GM-CSF (i.e. chronic idiopathic neutropenia, congenital neutropenia, sickle-cell-related neutropenia, autoimmune neutropenia). Neutrophil recovery was improved in most patients; however, no other clinical benefit or positive effect on survival was evident.^[206]

2.7 Leukaemia

Several large placebo-controlled trials have been performed with GM-CSF in leukaemia patients following induction chemotherapy. Neutrophil recovery was earlier in patients receiving GM-CSF. In one trial involving acute myelogenous

leukaemia, GM-CSF recipients^[209] time to achieve a neutrophil level of $>500/\text{mm}^3$ was 4 days shorter, and the incidence of infection was less (52 vs 75%) than in patients who received placebo. Achievement of a complete response was 69% in the GM-CSF-treated patients compared with 55% in the placebo-treated patients, although subsequent relapse occurred more frequently in the former (33 vs 14%) within the first 100 days after induction therapy. Overall survival duration was 378 days for patients receiving GM-CSF compared with 260 days in those receiving placebo. Other trials have reported similar effects on neutrophil recovery, but have not shown a survival advantage in patients who received GM-CSF.^[209-213]

3. Macrophage Colony-Stimulating Factor In Fungal Infections

M-CSF is a glycoprotein that stimulates survival, proliferation, and differentiation of mononuclear phagocytes.^[214] It also primes macrophages to enhance production of oxygen reduction products when stimulated by micro-organisms. As a result, M-CSF-treated monocytes have increased capability for intracellular killing of fungal and bacterial micro-organisms.^[215-219]

In order to evaluate its clinical potential and antimicrobial activities, M-CSF was administered to neutropenic and infected mice.^[215,220-224] Survival was significantly improved in mice infected with bacterial and fungal organisms compared with the results obtained from placebo, suggesting that M-CSF directly enhanced host resistance to infection by functionally activating monocytes.

The initial trial with M-CSF in patients with fungal infection was a phase I dose-escalation trial in which rhM-CSF was administered concomitantly with amphotericin B to 24 BMT patients with invasive fungal infection.^[225] Patients who received $\geq 2000 \mu\text{g}/\text{m}^2/\text{day}$ of M-CSF had a temporary reduction in platelet count by 61 000/mm³ from baseline while receiving the drug. GVHD in patients who had received allografts was not affected. Neutrophil, monocyte and lymphocyte counts were not altered.

Six patients had complete histological and radiological resolution of fungal infection during the study period. 12 patients were not evaluable for response (primarily because of refusal, or medical unsuitability, to undergo diagnostic surgical procedures for histological confirmation of infection resolution) and 6 patients did not respond to rhM-CSF. Two of the 6 patients who did not respond received less than 7 days of therapy, and 1 patient had an ANC of 0 and was unable to tolerate granulocyte transfusions. Ten of the 24 patients (42%) survived 100 days after initiation of therapy. No patients (10 with myeloid malignancy) developed recurrent disease while receiving M-CSF.

After completion of the phase I trial, 22 additional patients were treated with M-CSF at a dose of $2000 \mu\text{g}/\text{m}^2/\text{day}$.^[226,227] The results for all 46 M-CSF-treated patients were compared with those for 58 similar historical control patients (table VI). Patients with a Karnofsky score of $>20\%$ who received M-CSF and who had invasive *Candida* infection had better survival than historical controls.

Highly purified (not recombinant) M-CSF is indicated as therapy in Japan to accelerate granulo-

Table VI. Survival (percentage of total) of patients who received recombinant human macrophage colony-stimulating factor (rhM-CSF) compared with historical controls^[226]

Group	>20% Karnofsky performance score		$\leq 20\%$ Karnofsky performance score		Total
	<i>Candida</i>	<i>Aspergillus</i>	<i>Candida</i>	<i>Aspergillus</i>	
rhM-CSF	50 (n = 20) ^a	20 (n = 10)	0 (n = 11)	0 (n = 5)	27 (n = 46)
Controls	15 (n = 33) ^a	0 (n = 5)	9 (n = 11)	0 (n = 9)	5 (n = 58)
p-Value ^b	0.004	0.675	0.565	0.228	0.027

a Includes 1 patient with mucor who did not survive as a result of progressive infection.

b Mantel Cox analysis.

cyte recovery following allogeneic transplant, dose-intensive therapy of ovarian cancer and induction therapy of AML. In allogeneic transplant recipients, M-CSF was administered to 51 patients and the results were compared with concurrent nonrandomised controls. Two patients developed fever in association with the M-CSF infusion; otherwise there was no toxicity. The incidence and severity of GVHD, the rate of graft failure and the rate of recurrent disease and survival were not altered. Patients who received M-CSF for 14 daily doses achieved ANC_s of 500 and 1000/mm³ 4 ($p < 0.05$) and 8 days earlier,^[228-230] respectively, than control patients. M-CSF is administered as a short intravenous infusion at a dose of 8×10^6 IU/dose.

Ohno et al.^[231] recently completed a randomised, placebo-controlled trial in patients with AML. M-CSF ($n = 88$) or placebo ($n = 94$) were administered at a dose of 8×10^6 IU by 2-hour intravenous infusion for 14 days following consolidation chemotherapy. Patients receiving M-CSF completed all 3 chemotherapy courses a median of 17 days earlier than placebo-treated patients, as a result of more rapid neutrophil and platelet recovery. The duration of febrile neutropenia was also reduced in M-CSF-treated patients, from 10.4 to 6.4 days.

4. Conclusions

Both G-CSF and GM-CSF have established roles in minimising neutropenia-related complications following cytotoxic chemotherapy. The use of these cytokines, particularly G-CSF, has revolutionised the field of dose-intensive chemotherapy for the practising oncologist by permitting outpatient management of dose-intensive approaches through reduction of febrile neutropenic episodes and enhanced platelet recovery following infusion of cytokine-mobilised peripheral blood progenitor cells.

The results of this review suggest that G-CSF is the predominant cytokine utilised in oncology to reduce neutropenia-related complications associated with myelosuppressive chemotherapy. G-CSF is not as effective once neutropenia occurs, and it

does not appear to be effective in the setting of active infection when patients have normal or low neutrophil levels.

The field of transplantation has been dramatically altered with the use of peripheral blood progenitor cell infusions as a source of stem cells following aggressive myeloablative therapy. Based on a slightly improved tolerability assessment, G-CSF is also the cytokine of choice for mobilisation.

Data suggest that GM-CSF would be appropriately used in the setting of severe myelosuppression (possibly when the expected neutrophil level would be below 500 cells/mm³ for ≥ 7 days following dose-intensive chemotherapy). Patients appear to be at greater risk of infection during episodes of prolonged neutropenia, and the macrophage-stimulating effects of GM-CSF appear to reduce infection-related complications. GM-CSF is also an effective mobilisation agent, although modest bone pain and low-grade fever associated with GM-CSF make it a slightly more difficult cytokine to tolerate than G-CSF in this setting.

A benefit of either of these cytokines in the setting of infection without associated neutropenia has not been confirmed, although there is preliminary evidence of some efficacy.

Data suggest that M-CSF would be the most likely agent to be effective in the setting of fungal infection, whereas GM-CSF may be more appropriate in bacterial infection; however, these results are only preliminary and cannot be recommended for routine use.

Future trials will probably involve expansion of trials involving dose-intensive approaches with G-CSF. Future utilisation of GM-CSF will, most likely, involve situations of high infection risk. M-CSF, despite marked activity in enhancing monocyte and macrophage function, is not undergoing further investigation in the US.

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Erratum

Vol. 54, No. 2, page 234: The address for correspondence and reprints should have read: Dr Tsuneharu Baba, Vice-Director, Dohtai Clinic Kajiwara, 2-34-1 Kajiwara, Kamakura 247, Japan.

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